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PATHOLOGICAL AND VIRULENCE MARKERS OF TWO RARE *SALMONELLA* SEROVARS

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SUMMARY

Two rarely isolated *Salmonella* serovars, *Salmonella* Teshie (belonged to Arizona group) and *Salmonella* Ferruch were examined for selected virulence factors which may be operative in studied serovars including pathogenesis in mice, Congo red binding ability, production of heat-stable enterotoxin, cytotoxin, haemolysin and antibacterial resistance. The obtained results indicated that oral inoculation of mice with both serovars showed distribution of *Salmonella* in their different internal organs at different intervals ranging from, 3 hrs up to 9 days post inoculation associated with histopathological changes. Both serovars had Congo red binding activity. *S.* Ferruch but not *S.* Teshie produced heat-stable enterotoxin. Both serovars were not cytotoxic to Vero cells. Moreover, both serovars produced (-haemolysin, and had multiple antibacterial resistance.

INTRODUCTION

Infection with *Salmonella* in animals has a major economic effects as well as the transmission of the infection to human.

Salmonellae were responsible for several outbreaks in bovines in different parts of the world causing abortion or other diseases ranging from gastroenteritis to septicaemia with mortality in some cases depending on the serovar of the bacterium and the nature of the infected host (Hinton, 1972).

Salmonella infection is usually associated with inadequate nutrition, poor hygienic measures or any other stress factors (Steenkamer, 1966).

For the importance of *Salmonella* as a zoonotic disease, its public health and animal wealth haz-

ards, this research was therefore, carried out to explore if the two rarely isolated *Salmonella* serovars (*S.* Ferruch and *S.* Teshie) have the same importance as the other serovars of *Salmonella*. This was achieved through the study of certain characteristics that might be virulence markers including:

* Pathogenesis of *Salmonella* serovars Teshie and Ferruch was studied in mice by oral infection and re-isolation in addition to histopathological examination of different organ tissues for detection of pathological effects in internal organs of the inoculated mice at different intervals.
* Congo red binding ability.
* Baby mice assay for enterotoxin Production.
* Vero cell cytotoxicity.
* Production of haemolysin.
* Antibacterial resistance pattern.

MATERIAL AND METHODS

Bacterial serovars:

Two *Salmonella* serovars Ferruch and Teshie were studied. The first serovar, *S. Ferruch* was isolated by Amal Ghoniem (ARRI) and serotyped at Animal Health Research Institute; this serovar was isolated from milk and internal organs of newly born calves suffered from salmonellosis and died suddenly as well as from other mortalities in the same dairy farm. Whereas the second serovar Teshie belonged to Arizona group was isolated from raw meat by Saad et al. (1998) and was serotyped at the Central Laboratory of Ministry of Health, Egypt.

Bacteriological tests and serological typing of the isolated *Salmonella* serovars were carried out according to Kauffman White scheme (Cruickshank et al (1975); Koneman et al (1983); Varnam and Evans (1991).

Pathogenesis of *Salmonella*:

This test was carried out according to Weinsten et al. (1984) in which seventy seven albino mice were used in this experiment, five mice of which were kept separately as a control group and the other seventy two were divided into two groups. Each group was inoculated with one serovar of *Salmonella*.

Mice of each tested group were deprived of water overnight and were given 25 (1 2 x 108 of 18 hours broth culture of *Salmonella*.

The mice were left for 9 days post infection. Three mice from each inoculum were sacrificed at different intervals; 3 hrs, 6 hrs, 12 hrs, 24 hrs, 2 days, 3 days and daily up to the 8th day. The remaining mice were died at the 9th day post infection.

The internal organs of the inoculated mice including heart, liver, spleen and intestine were divided into two portions, one part was used for bacterio-

logical examination of *Salmonella*, whereas the second part was put into 10% neutral buffered formalin for histopathological examination.

Bacteriological examination of internal organs of the inoculated mice:

The liver, spleen, heart and intestine of the inoculated mice were bacteriologically examined. All samples were cultured for detection of *Salmonella*.

Each organ parts were immersed in 70% ethyl alcohol, flammed, cut aseptically into small portions and inoculated into buffered peptone water for 24 hrs at 37°C. The culture was inoculated in selenite F broth for 18 hrs at 37°C. The culture was then streaked onto S. S. agar and Endo agar plates, incubated at 37°C for 24 hrs. Suspected colonies were picked up. The obtained pure cultures from separate colonies were subjected to biochemical tests (Koneman et al., 1983).

Congo red binding ability test (Berkhoff and Vinal, 1986):

S. Ferruch *and* S. Teshie were grown onto Congo red agar plates. The inoculated plates were incubated at 37°C for 24 hrs, and then left at room temperature for another 48 hrs and not exceed 4 days. Congo red positive serovars developed red colonies, whereas, Congo red negative organisms showed white colonies.

Baby mice assay for Detection of heat-stable enterotoxin production (Guarino et al., 1987):
Baby mice assay was used for detection of the ability of the tested *Salmonella* serovars to produce heat-stable enterotoxins, this was through the intragastric inoculation of suckling mice (1 to 3 days old) with 0.1 ml of the prepared tested toxin from *Salmonella* serovars. The inoculated mice were sacrificed after four hrs post inoculation. The ratio between intestinal weight to the remaining body weight was calculated and the intestines were examined for distension.

Vero cell cytotoxicity assay (Emery et al., 1992):

The ability of both *Salmonella* serovars to produce cytotoxin was examined through inoculation of the prepared culture supernatants from *Salmonella* serovars into Vero cells, and the inoculated Vero cell line was examined for the presence of cytopathic effects. The cytopathic effect was examined at 12, 24, 48 and 72 hrs intervals. The degree of cytopathic effect was recorded as 0, 1, 2, 3 or 4 degrees corresponding to 0 to 25%; 25 to 50% ; 50 to 75%; 75 to 90% and 90% or more of the Vero cell changes.

Detection of haemolytic activity. (Koneman et al., 1983):

Both *S.* Ferruch and *S.* Teshie were grown onto sheep blood agar plates, incubated at 37°C for 24 hrs and examined for the presence of α or β haemolysis.

Antibacterial susceptibility test:

This test was carried out on both serovars *S.* Ferruch and *S.* Teshie according to Koneman et al. (1983) and Quinn et al. (1994) using the antibacterial agents shown in Table (1):

Histopathological examination (Carlton, 1976):

Specimens from heart, intestine, liver and spleen of the inoculated mice with *Salmonella* serovars were collected immediately after sacrification of the inoculated mice, and were taken in 10% neutral buffered formalin. The fixed specimens were then prepared as 5 micron thick paraffin sections and stained with Haematoxalin and Eosin (H & E) for microscopic examination.

Table (1): Antibacterial agents

Antibacterial agents	symbol	concentration (μg)
Ampicillin	AMP	10
Chloramphenicol	C	30
Ciprofloxacin	Cip	5
Gentamicin	GM	10
Neomycin	N	30
Norofloxacin	NoR	10
Streptomycin	S	10
Trimethoprim - sulphamethoxazol 1.25 + 23.75 (SXT)		

*The antibacterial discs were abtained from Oxoid Co.

RESULTS

II. Histopathological findings:

1. Intestine:

The intestinal lesions were recorded throughout the experimental period. Intestine of mice infected with *S.* Ferruch revealed sloughing of its epithelial lining, hyperactivity of intestinal glands and mononuclear cell infiltration. In case of mice group inoculated with *S.* Teshie, the pathological findings were necrosis of intestinal villi and glands accompanied with mononuclear cells aggregation (Fig.1).

2. Liver:

Liver of mice inoculated with *S.* Ferruch showed mild degenerative changes at the 1st day of the experiment. Centrolobular necrosis of hepatocytes was observed at the 5th day of inoculation (Fig.2). Necrobiotic changes, hyperplasia of bile duct with mononuclear cells aggregation were seen at the end of experimental period (Fig.3). Liver of mice infected with *S.* Teshie showed swelling of hepatocytes at the 1st day of infection, while massive necrobiotic changes of hepatocytes at the 5th day post inoculation were seen (Fig.4). Focal mononuclear cell aggregation at the portal area with telangectasis were seen at the end of experiment (Fig.5).

Table(2): Serotyping and antigenic structure of the studied *Salmonella* serovars.

Salmonella serovar	Group	Antigenic structure		
		Somatic (0)	Flagellar (H)	
			Phase 1	Phase 2
S. Ferruch	C_3	8	e,h	1,5
S. Teshie	X	1, 47	1, Z_{13}, Z_{28}	e, n , Z_{15}

Table (3): Congo red binding activity of *Salmonella* serovars.

Salmonella serovar	Binding activity to Congo red
S. Ferruch	+ ve
S. Teshie	+ ve

Table (4): Enterotoxigenic activity of *salmonella* serovars using Baby mice assay.

Salmonella serovar	Inoculated suckling mice			Results
	Intestinal weight	Remaining body weight	*Ratio	
S. Ferruch	0.2511	2.850	0.088	+ ve**
S. Teshie	0.2274	2.958	0.0768	-ve

* Ratio between intestinal weight to the remaining body weight.

** Positive result was more than 0.083

Table (5): Antibacterial susceptibility test.

Antibacterial agents	Concentration (µg)	Sensitivity of *Salmonella* to the antibacterial agents	
		S. Ferruch	*S.* Teshie
Ampicillin (AMP)	10	sensitive	sensitive
Chloramphenicol (C)	30	Resistant	Resistant
Ciprofloxacin (Cip)	5	sensitive	sensitive
Gentamicin (GM)	10	Resistant	Resistant
Neomycin (N)	30	Resistant	Resistant
Norofloxacin (NoR)	10	sensitive	sensitive
Streptomycin (S)	10	Resistant	Resistant
Trimethoprim	1.25+	Resistant	Resistant
sulphamethoxazol (SXT)	23.75		

3- Spleen:

Spleen of mice inoculated with *S.* Ferruch, showed depletion of lymphoid follicles and vascular oedema (Fig. 6). Haemorrhage, eosinophilic oedematous fluid, replacing the splenic parenchyma were seen in mice inoculated with *S.* Teshie. Also megakaryocytes were observed (Fig.7).

4- Heart:

Heart of mice inoculated with *S.* Ferruch showed mild degeneration of cardiac muscle. In case of *S.* Teshie infection, focal hyalinosis of cardiac muscle was seen (Fig.8).

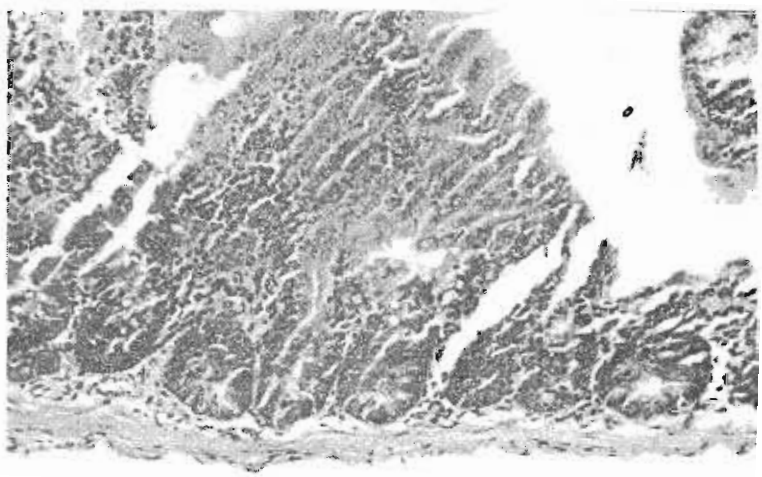

Fig. (1): Intestine of mice infected with S. Teshie showing necrosis of intestinal villi with mononuclear cells infiltration (H & E X 200).

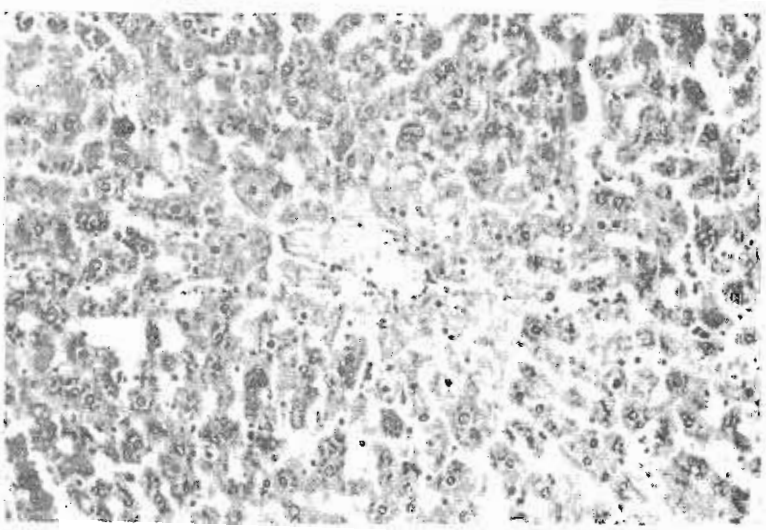

Fig. (2): Liver of mice infected with S.Ferruch (5th day of infection) showed centrolobular necrosis of hepatocytes (H & E X 200).

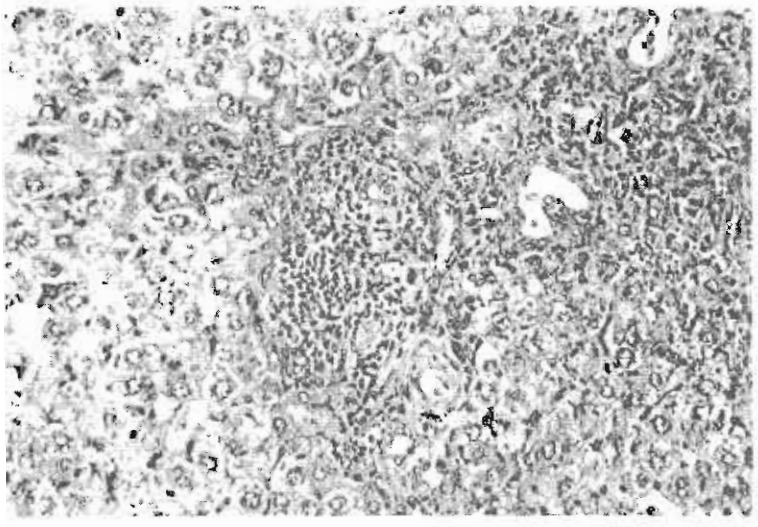

Fig. (3): Liver of mice infected with S. Ferruch (8th day of infection) showing hyperplasia of bile duct and focal mononuclear cells aggregation (H & E X 200).

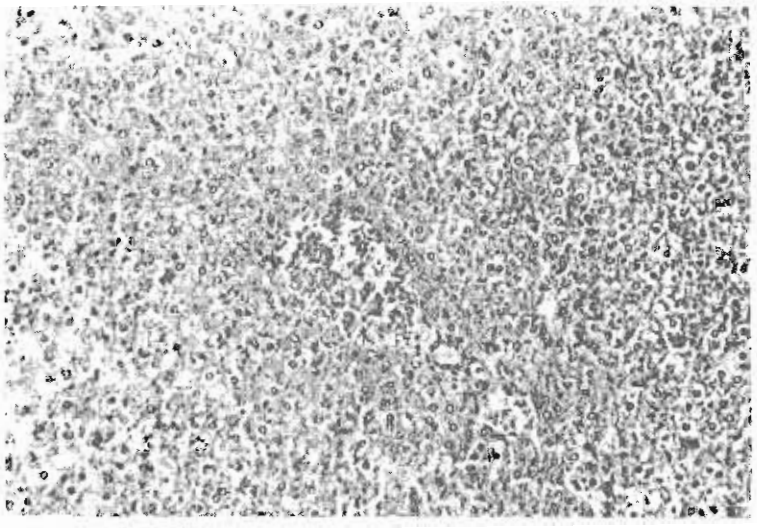

Fig. (4): Liver of mice infected with S.Teshie (5th day of infection) showing diffuse, extensive necrobiotic changes of hepatocytes (H & E X 100).

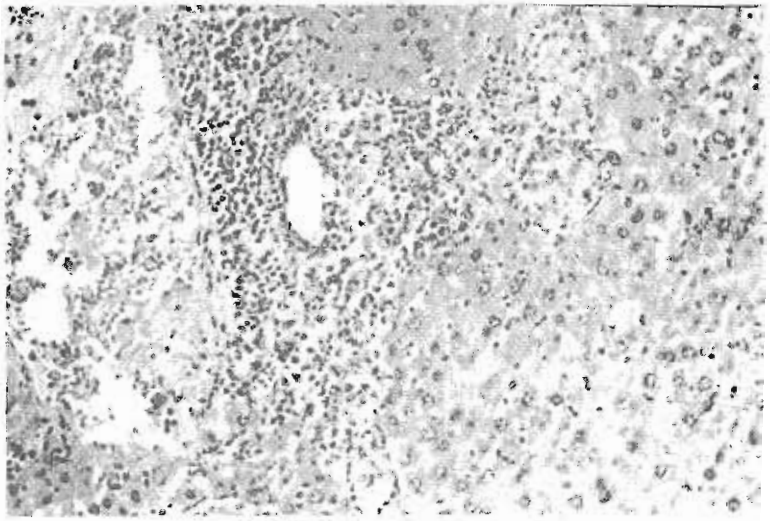

Fig. (5): Liver of mice infected with S. Teshie (8th day of infection showing focal mononuclear cells aggregation and telangectasis of blood vessels (H & E X 100)

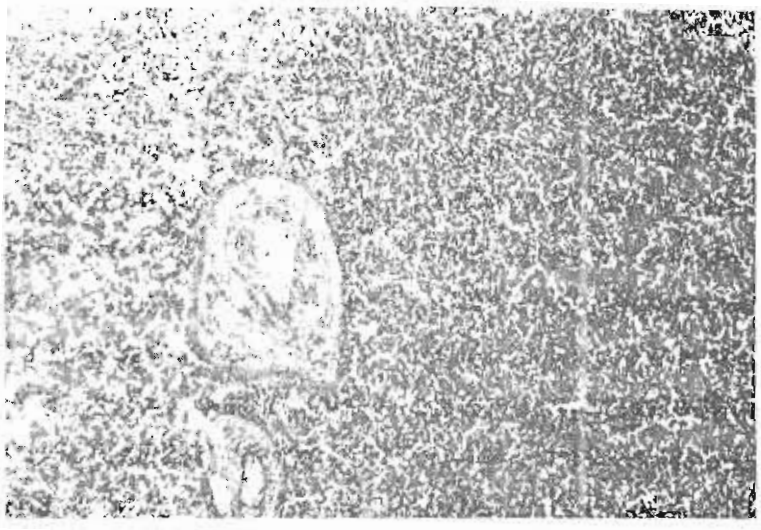

Fig. (6): Spleen of mice infected with S. Ferruch showing depletion of lymphoid follicles and vascular oedema (H & E X 100).

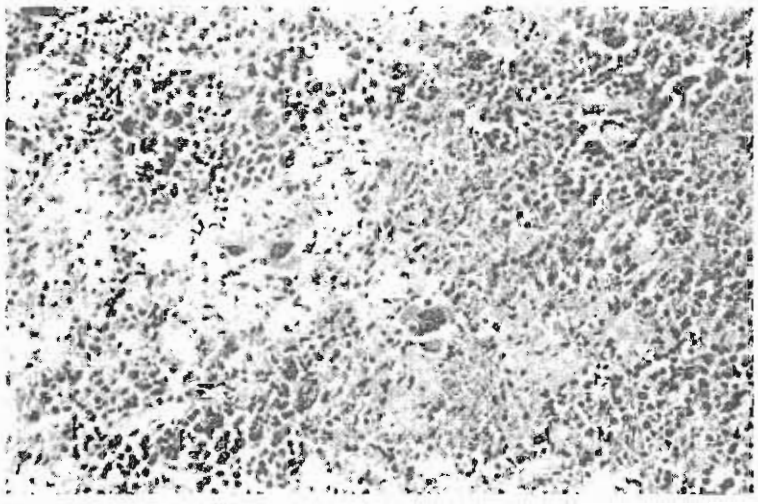

Fig. (7): Spleen of mice infected with S. Teshie showing haemorrhages, oedema with prominant megakarocytes (H & E X 200).

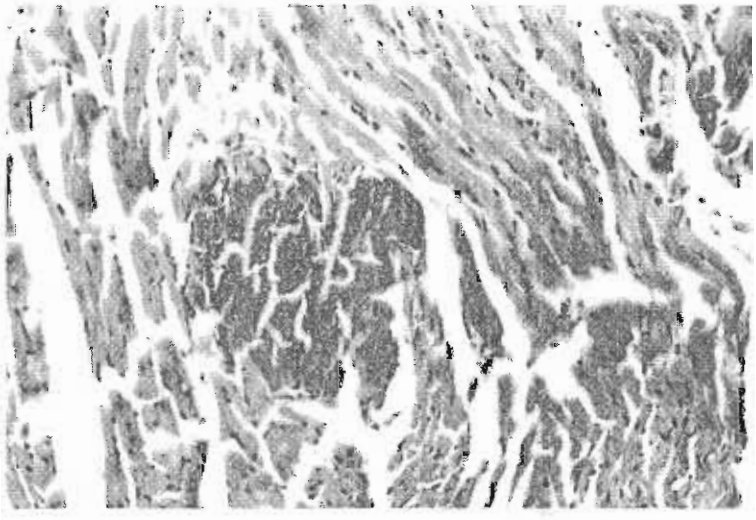

Fig. (8): Heart of mice infected with *S.* Teshie showing focal hyalinosis of cardiac muscles (H & E X 100).

DISCUSSION

Salmonella infection is of public health importance concerned with human and animals. It is considered to be one of the major zoonotic diseases (Farid et al., 1987).

In an attempt to clarify the early pathogenesis of *Salmonella*, it was found that the organism invades the epithelium of the small intestine through their brush border or intracellular junction (Boyd, 1990). This process occurs through time which could be detected as early as 2 hrs with adverse changes along with time after 12, 18 hrs as shown with *S.* Typhimurium (Varnam an Evans, 1991).

The ability of orally ingested *Salmonella* serovars to establish systemic infection is evident by detection of the organisms in the intestine and liver by the third hour post infection. The most evident difference between *Salmonella* serovars Teshie and Ferruch is the ability of the former to invade and multiply in heart blood and spleen as early as 3 hrs. Inoculated mice were died within 9 days post infection.

Histopathological examination of intestine of inoculated mice with *S.* Ferruch showed sloughing of lamina epithelialis and hyperactivity of intestinal glands. This type of mild enteritis is similar to that caused by *S. Anatum*, S. Newport, S. Reading and S. Meleagridis as was reported by Flott et al. (1981) and Jones and Hunt (1983). The intestinal mucosa of infected mice with *S. Teshie* showed necrosis of intestinal villi and focal aggregation of mononuclear cells. These findings are in accordance with those described by Muir (1992) who reported the same lesion in case of infection with S. Paratyphi.

The histopathological alterations of liver tissues of infected mice with *S.* Ferruch showed mild degenerative changes followed by centrolobular necrosis of hepatocytes and hyperplasia of bile duct with mononuclear cells aggregation at the end of the experiment. The suggestion of centrolobular necrosis of liver may be resulted from destruction of the efferent hepatic vessels due to endothelial damage end by thrombosis under the effect of salmonella endotoxin.

On the other hand, the hepatic tissue of mice infected with *S.* Teshie showed massive necrobiotic changes of hepatocytes. Later on, focal aggregation of histeocytes specially in the portal traids and telangiectasis were seen, these findings agreed with those described by Boyd (1990) and Jubb et al. (1991) who recorded that, the liver infected with *S.* Paratyphi revealed necrobiotic changes of hepatocytes in addition to the presence of paratyphoid nodules which mostly similar

to that observed in *S. Teshie* infection.

In spleen, *S.* Ferruch could be detected at the 7th day of inoculation, although, S. Teshie could be isolated from the spleen of the inoculated mice at 3 hrs intervals and the ninth day post infection. Spleen of infected mice with *S.* Ferruch showed depletion of white pulp and vascular oedema, while in case of *S. T*eshie infection haemorrhages, oedema with permanent megakaryocytes were observed. These findings may be resulted from the effect of certain *Salmonlla* serovars (Mc Gavin et al., 2001).

Although, *S.* Teshie could be detected from 3 hrs after mice inoculation, *S.* Ferruch was detected only in heart at the 7th day of mice inoculation. The heart showed mild degenerative changes in case of mice infected with *S.* Ferruch, while heart of mice infected with *S.* Teshie showed multiple focal hyalinized areas. The necrobiotic changes of the cardiac muscle may be due to *Salmonella* endotoxins.

The presence of salmonella serovars in different internal organs of orally inoculated mice could be attributed to systemic spread of Salmonella, the possible route of the invasion and multiplication of *Salmonella* in the lymphatic system from which the organism spreads to all parts of the body.

Salmonella causing infection, usually have one or more virulence factors that may enable the organism to be established at the site of infection, invades, survives and multiplies. One of these virulence factors is the Congo red binding activity. It is worthy to mention that both S. Ferruch and S. Teshie could bind Congo red dye (100%). The property of the organism to bind Congo red dye is correlated with the invasiveness of the bacteria (Maurelli et al., 1984 and Qadri et al., 1988) and also associated with pathogenicity and virulence of *Salmonella* (Albert, 1991, and Rombling et al., 1998).

The enterotoxigenic activity of *Salmonella* serovars exhibited through baby mice assay showed that S. Ferruch but not S. Teshie had the ability to produce heat-stable enterotoxin.

Non of *Salmonella* serovars studied gave cytotoxic activity on Vero cells, this may be due to the fact that the role of *Salmonella* enterotoxins to express the disease condition is far from clear, and it is affected by complex factors including host and the organism itself which sometimes could not occur in vitro (Wallis et al., 1986).

Blood haemolysis is also one character of virulent miocroorganisms (Koneman et al., 1983), from the obtained results it was found that both S. Ferruch and *S.* Teshie were (β haemolytic serovars

indicating their virulence.

The results of antibacterial susceptibility test as shown in table (4) showed multibacterial resistance of both *Salmonella* serovars as they were resistant to chloramphenicol, gentamicin, neomycin, streptomycin and trimethoprim sulphamethoxazol. Whereas both serotypes were sensitive to ampicillin, ciprofloxacin and norofloxacin.

The multidrug resistance of *Salmonella* is a serious problem due to difficulties in their treatment, as well as the possible transmission of antibiotic resistance to other enteric bacteria through the transmission of antibiotic resistant plasmid (Tassios et al., 1997) with no response to different therapeutic drugs.

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